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(54) Title: TRYPTOPHAN ANALOGUES AS SELECTIVE AGENTS IN THE TRANSFORMATION OF PLANTS AND PLANT CELLS

(57) Abstract

The instant invention provides a method for the selection of transformed plant cells, comprising the steps of: i) transforming plant cells with a polynucleotide sequence comprising a plant expressible gene encoding a tryptophan analogue converting activity, ii) culturing said plant cells, or growing plant material comprising said plant cells, in the presence of a selective amount of a tryptophan analogue deleterious to non-transformed plant cells, under conditions that allow for the manifestation of said deleterious effect.

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Tryptophan analogues as selective agents in the transformation of plants and plant cells

TECHNICAL FIELD

The present invention is related to the selection of transformed plant cells or plants. The invention also comprises plants obtained by the said method as well as DNA sequences useful therein.

BACKGROUND OF THE INVENTION

In the process of introducing a useful property into plants or plant cells by genetic engineering, it has proven almost unavoidable to make use of a selection marker. This necessity is partly related to the fact that many valuable properties can not be readily observed during the transformation process and the fact that transformation frequencies are still rather low.

If the marker gene and the DNA sequence of interest providing the useful property are located on the same transforming polynucleotide sequence the introduction of the marker gene is likely to coincide with the introduction of the gene of interest. Hence, a first selection for cells that contain and express the marker will generally reduce the number of cells that have to be analysed for the presence and expression of the gene of interest. Obviously, this saves laboratory space, working hours and reduces costs considerably; in many cases, especially with the more difficult crops, transformation without selection is not feasible at all.

Among the marker genes that are most widely used in plant transformation are the bacterial neomycin phosphotransferase genes (nptI, nptII and nptIII genes) conferring resistance to the selective agent kanamycin, disclosed in EP-B 131 623, and the the bacterial aphIV gene, disclosed in EP-A 186 425 conferring resistance to hygromycin.

These markers are not suitable for selection of transformants of all plant species; some plant species appear to be naturally resistant against the selective agents used

herein (Waldron, C. et al., (1985) Plant Molecular Biology 5, 103-108; Van den Elzen, P.J.M. et al., (1985) Plant Molecular Biology 5, 299-302).

Eukaryotic genes are also known to be used as selection markers in plants. EP-A 256 223 discloses a Glutathion-S-transferase gene from rat liver, conferring resistance to glutathion derived herbicides.

Overproduction of yeast glutamin synthetase confers resistance to glutamine synthetase inhibitors such as phosphinotricin, as disclosed in WO87/05327.

EP-A 275 957 discloses the use of an acetyl transferase gene from Streptomyces viridochromogenes that confers resistance to the selective agent phosphinotricin.

Plant genes conferring relative resistance to the herbicide Glyphosate are disclosed in EP-A 218 571. The resistance is based on the expression of a gene encoding a 5-enolshikimate-3-phosphate synthase (EPSPS) that is relatively tolerant to N-phosphonomethylglycine. The selective compound is not converted by EPSPS.

In spite of the existence of a number of selectable marker genes that are suitable for plant species there is still a need for other selectable marker genes.

STATE OF THE ART

Tryptophan analogues have been used to screen for Catharanthus roseus cells with a high endogenous tryptophan decarboxylase (TDC) activity (Sasse F. et al., (1983) Z. Naturforschung 38c, 910-915). The analogues with the most growth inhibitory activity were identified as 4-methyl-, 4-fluoro-, 5-fluoro-, and 5-hydroxy-tryptophan. Cultured Catharanthus cells with increased resistance to 4-mT contained increased levels of TDC. Therefore, the authors assumed that it should be possible to devise selection schemes for establishing plant cell cultures with higher activities of detoxifying enzymes with the purpose of obtaining strains with the capacity to produce increased levels of useful secondary metabolites.

The complete DNA sequence of a cDNA encoding the tryptophan decarboxylase (EC 4.1.1.28, formerly 4.1.1.27) of Catharanthus roseus has been disclosed (De Luca V. et al., (1989) Proc. Natl. Acad. Sci. USA 86, 2582-2586). The cDNA encodes a protein of 500 amino acids. It was shown that this cDNA can be expressed in transgenic tobacco plants under the control of the CaMV 35S promoter (Songstad et al., (1990) Plant Physiol. 94, 1410-1413). Transgenic tobacco plants showed up to 260 times increased tryptamin levels due to the conversion of endogenous tryptophan. These plants seemed phenotypically normal. This suggests that plants expressing increased levels of TDC may be useful for producing (increased levels of) commercially important antineoplastic monoterpenoid indole alkaloids, vinblastine and vincristine, as suggested in International Patent Appliction, WO90/10073 published on 7 September 1990.

Tryptophan analogues have never been used for the selection in the process of transforming plant cells and plants. The use of a plant expressible TDC gene as a selectable marker gene in a process of transforming plants has not been disclosed.

SUMMARY OF THE INVENTION

The invention provides a method for the selection of transformed plant cells comprising the steps of

i) transforming plant cells with a recombinant polynucleotide comprising a plant expressible gene encoding a tryptophan analogue converting activity,

ii) culturing said plant cells, or growing plant material comprising said plant cells, in the presence of a selective amount of a tryptophan analogue deleterious to non-transformed plant cells, under conditions that allow for the manifestation of said deleterious effect.

A preferred plant expressible gene is one encoding tryptophan tryptophan decarboxylase. Still further preferred is a tryptophan decarboxylase gene from Catharanthus roseus or a functional derivative thereof. Most preferred is a plant

expressible decarboxylase gene under the control of the CamV 35S promoter.

According to a preferred embodiment of the invention the said recombinant polynucleotide further comprises a polynucleotide sequence of interest which is a plant expressible gene giving rise to fungal resistance, insect resistance, nematode resistance, virus resistance, altered carbohydrate composition, altered oil composition, altered amino acid composition, male-sterility, modified flower color, modified fruit ripening, salt resistance, herbicide resistance, antibiotic resistance, production of a secondary metabolite, production of a pharmaceutical protein, or production of enzymes useful in an industrial process.

Preferred for use as selective agents in a method according to the invention are tryptophan analogues selected from the group consisting of 4-methyltryptophan (4-mT), 5-methyltryptophan (5-mT), 4-fluorotryptophan (4-fT) and 5-hydroxytryptophan (5-hT).

The invention also comprises plant cells obtained by a method according to the invention, as well as plant material and plants harbouring such cells. Preferred plant parts are those selected from the group consisting of bulbs, flowers, fruits, hairy roots, leaves, microtubers, pollen, roots, seeds, stalks and tubers.

A further embodiment of the invention comprises the use of a tryptophan analogue according to the invention for the selection of a transformed plant cell.

Yet another aspect of the invention comprises the use of a plant expressible gene encoding an enzyme having a tryptophan analogue converting activity as a marker gene for the selection of transformed plant cells.

The invention further comprises a method for obtaining a transformed plant comprising the steps of:
i) transforming plant cells with a polynucleotide sequence comprising a plant expressible gene encoding a tryptophan analogue converting activity,
ii) culturing said plant cells, or growing plant material

comprising said plant cells, in the presence of a selective amount of a tryptophan analogue deleterious to non-transformed plant cells, under conditions that allow for the manifestation of said deleterious effect,

iii) regenerating surviving cells of step ii) into a plant,

iv) identifying a transformed plant.

Preferred in this method is a plant expressible gene encoding a tryptophan analogue converting activity is a plant expressible tryptophan decarboxylase gene. Still more preferred in the method is a tryptophan decarboxylase gene from Catharanthus roseus or a functional derivative thereof. In a still further preferred embodiment the tdc gene is under the control of the CaMV 35S promoter.

In a highly preferred embodiment said recombinant polynucleotide further comprises a polynucleotide sequence of interest. More preferably said polynucleotide sequence of interest is a plant expressible gene which gives rise to fungal resistance, insect resistance, nematode resistance, virus resistance, altered carbohydrate composition, altered oil composition, altered amino acid composition, male-sterility, modified flower color, modified fruit ripening, salt resistance, herbicide resistance, antibiotic resistance, production of a secondary metabolite, production of a pharmaceutical protein or production of an industrial enzyme.

Another embodiment of the invention is a plant obtained with a method according to the invention, as well as progeny plants obtained after sexually or asexually propagating said plants.

The invention also comprises a product obtained after the processing of a plant part of plants obtained with a method according to the invention.

Another aspect of the invention is a recombinant polynucleotide which can be used for the transformation of plant cells and subsequent selection of transformed plant cells, comprising a plant expressible gene encoding a tryptophan analogue converting activity. Another embodiment of the invention comprises said recombinant polynucleotide

which further comprises a polynucleotide sequence of interest, with the proviso that said polynucleotide sequence of interest is not a plant expressible nptII gene. Still further preferred is a recombinant polynucleotide with the proviso that said polynucleotide sequence of interest is not known as a selectable marker gene for use in the transformation of plants. A preferred recombinant polynucleotide according to the invention is one wherein the polynucleotide sequence of interest comprises a plant expressible gene which gives rise to fungal resistance, insect resistance, nematode resistance, virus resistance, altered carbohydrate composition, altered oil composition, altered amino acid composition, male-sterility, modified flower color, modified fruit ripening, salt resistance, production of a secondary metabolite, production of a pharmaceutical protein or production of an industrial enzyme.

A different aspect of the invention comprises a recombinant plant DNA genome containing a copy of a recombinant polynucleotide according to the invention, as well as plants or plant cells containing such recombinant plant DNA genome.

The invention also comprises a substantially pure DNA molecule which comprises the nucleotide sequence represented in SEQIDNO: 2.

The advantages and the field of application will be readily appreciated from the following detailed description of the invention.

DESCRIPTION OF THE FIGURES

The following figures further illustrate the invention.

Figure 1: A diagramatic representation of the cloning steps resulting in the binary vector pBDH5, containing the nptII gene under control of the Nos promoter and terminator; the binary vector pTDCs containing the nptII gene and the tdc sense construct; the binary vector pTDCa

containing the nptII gene and the tdc antisense construct;

Figure 2: Northern blot: Analysis of tdc transcript levels in transgenic Nicotiana tabacum plants obtained after leaf-disc transformation with LBA 4404 containing pBDH5 (vector), pTDCa (antisense), or pTDCs (sense) constructs. The blot was hybridised with ^{32}P dCTP labelled tdc cDNA: A - J represent different independent lines harbouring either the sense constructs (SENSE A - J), the antisense constructs (ANTISENSE A - D) or the 'empty' vector pBDH5 (VECTOR A - B). SENSE constructs were classified as low (A, G), intermediate (B, C, D, F) or high expressors (E, H, I, J).

Figure 3: Leaf-discs of pTDCs transgenic N. tabacum plants cultured on shooting medium containing 0, 0.05, 0.10, 0.50 or 1.00 mM 4-methyl tryptophan. Leaf explants were used from tobacco plants A, C and I containing respectively low (sense: L), intermediate (sense: M) and high (sense: H) tdc transcript levels. As a control, leaf explants from a pBDH5 (vector) transformed plant were used.

Figure 4: Leaf-discs of N. tabacum treated with LBA 4404 containing pTDCs (sense), pTDCa (antisense), pBDH5 (vector) and leaf-discs not treated with Agrobacterium (- Agrob). Selection was performed on 0.1 mM 4-methyl tryptophan.

Figure 5: Northern blot: tdc and nptII transcript levels in transgenic plants obtained after leaf-disc transformation with LBA 4404 containing pTDCs

(sense) and subsequent selection on 0.1 mM 4-methyl tryptophan.

DETAILED DESCRIPTION OF THE INVENTION

The present invention discloses a method for the selection of transformed plant cells, comprising the steps of:
i) transforming plant cells with a polynucleotide sequence comprising a plant expressible gene encoding a tryptophan analogue converting activity,
ii) culturing said plant cells, or growing plant material comprising said plant cells, in the presence of a selective amount of a tryptophan analogue deleterious to non-transformed plant cells, under conditions that allow for the manifestation of said deleterious effect. The various aspects of the invention are further clarified below. Such aspects concern 1) the plant expressible marker gene, 2) the selection conditions, 3) the plant material to be transformed 4) transformation of plant material with the said polynucleotide sequence 5) the polynucleotide sequence of interest.

For a better understanding of the various ways of practicing the invention a number of these aspects will be outlined in more detail below. The enumeration is not meant to be limitative with respect to ways of carrying out the invention, its applicability or in any other way.

Whenever the expression 'tryptophan analogue' is used reference is made to tryptophan itself or a compound having a alkyl-, hydroxyl-, halo-, aryl-, aryloxy-, alkoxy-, or aza-group in the position 1, 4, 5, 6, or 7, or any combination of two or more of such groups.

1) the plant expressible marker gene

The expression 'plant expressible marker gene' refers to a polynucleotide sequence comprising the marker gene as well as the regulatory sequences required for expression of the marker gene in the plant cell.

Suitable marker genes that fall within the scope of the

invention are those which encode an enzyme having the capacity to convert a tryptophan analogue according to the invention into a different analogue with a less toxic effect on the plant cell. Such enzymes are not limited to decarboxylases; any other enzymatic activity having the capacity to convert a toxic tryptophan analogue are useful as long as the conversion results in detoxification of the said tryptophan analogue.

The word 'gene' as used here is meant to comprise cDNAs as well as genomic clones, as well as synthetic or partially synthetic analogues thereof that encode a protein; they may be derived from procaryotes and eucaryotes alike.

The regulatory sequences may include promoters and so-called enhancers, which may drive expression constitutively or developmentally and/or environmentally regulated. Many promoters that are generally suitable for the expression of genes in plants are described in the prior art. In order to be useful to drive expression of the marker gene according to the invention it is necessary that the promoter is functional in the plant cell during application of selection pressure. Therefore, promoters generally regarded as constitutive are preferred, such as the CaMV 19S promoter and the CaMV 35S promoter, or the promoters derivable from the T-DNA of Ti-plasmids from Agrobacterium, although any other homologous or heterologous promoter that meets the requirements set out above may be used. It will be understood by those skilled in the art that promoters obtainable from endogenous plant genes are suitable as well.

The selectable marker gene will generally comprise a so-called terminator sequence, including a polyadenylation signal, for proper expression of the marker gene. Said terminator may be homologous or heterologous to the said gene. Sources of suitable terminators sequences are well known to those of skill in the art.

2) the selection conditions

The selection conditions may vary depending on for

instance the choice of the tryptophan analogue and the choice of the plant material used.

Obviously, tryptophan analogues that are less toxic to a particular plant cell may need a higher concentration to obtain an effective selection of a transformed cell, whereas those that are more toxic require a lower concentration. Likewise a higher concentration of the trytophan analogue may be used if the plant material to be transformed is less susceptible to the analogue, or if it is obtained from a plant already showing some degree of tryptophan analogue converting activity.

An amount of a tryptophan analogue is said to be selective if it is capable of reducing the increase of fresh weight of plant cells as compared to the increase of fresh weight in medium to which no tryptophan analogue has been added. Preferably, the selective amount of the tryptophan analogue effectively kills all non-transformed cells while transformed cells are not affected at all. In practice such a situation will rarely exist and for each type of plant material the optimal conditions have to be determined in which the increase of fresh weight of non-transformed plant material is inhibited as much as possible while the toxic effect to transformed cells remains within acceptable limits. Obviously, when transformed cells should give rise to whole plants, the regenerative capacity of the cells must not be affected by the concentration of the selective agent used.

Tryptophan analogues which have a toxic effect on plant cell growth are inter alia L-tryptophan, D-tryptophan, α-methyltryptophan, N-methyl-L-tryptophan, 1-methyltryptophan, 4-methyltryptophan, 5-methyltryptophan, 6-methyltryptophan, 4-fluorotryptophan, 5-fluorotryptophan, 6-fluorotryptophan, 5-hydroxytryptophan, 5-methoxytryptophan, 5-benzyloxytryptophan and 7-azatryptophan. Whether or not a tryptophan analogue can in fact be converted by TDC from Catharanthus roseus can be determined by a number of techniques, the choice of which is not crucial to the invention; one such method is disclosed by Sasse et al.,

(1983), supra. If the tryptophan analogue is found to be converted by TDC the tdc gene from Catharanthus roseus can be used as marker gene. If desired a tryptophan decarboxylase gene may be used from another plant or even from non-plant origin as long as it encodes a tryptophan decarboxylase capable of converting and (partly) detoxifying the used tryptophan analogue. Different plant species may produce a tryptophan decarboxylase with a different substrate specificity as compared to the TDC from Catharanthus roseus.

If desired, an enzymatic activity different from a decarboxylase may be selected, as long as the tryptophan analogue is converted into a non- or less toxic compound. A gene encoding the said enzymatic activity can be used as selection gene using a tryptophan analogue that can be converted by the said enzymatic activity.

Especially preferred as selective agent is the tryptophan analogue 4-methyltryptophan, preferably in a concentration range between 0.1 and 0.5 mM. More preferably, said selective agent is used in combination with a tryptophan decarboxylase gene as marker gene. Most preferably said tryptophan decarboxylase gene is a tdc gene derived from Catharanthus roseus.

Determination of the optimal conditions such as the selective amount and stage of exposure for each tryptophan analogue or plant material used should be well within the skill of the average worker in the area of technology to which this invention pertains.

3) The plant material to be transformed

For the purpose of this invention the expression 'plant' is not limited to species used in agriculture, floriculture or horticulture, but also includes such species used in activities such as gardening, forestry and the like.

The plant material used in the transformation process may vary due to inter alia the plant species to be transformed, the method of transformation, the nature of the plant material, such as protoplasts, cultured cells, pollen, leaf

tissue, embryonic tissue and the like, origin of the plant material, e.g. monocotyledonous or dicotyledonous plants, the necessity and the capacity of regenerating the plant material in a full grown transformed plant and the like. For each plant material the sensitivity to a particular tryptophan analogue may be determined by making a so-called killer curve; the optimal tryptophan analogue may be selected by comparing different analogues at a fixed concentration and subsequently selecting the optimal analogue. The optimal concentration of the analogue of choice may be determined by testing a concentration range, optionally in different stages of the selection process.

4) transformation of plant material with the said polynucleotide sequence

The expression transformation with a polynucleotide sequence refers to the various ways of introducing a recombinant polynucleotide directly or indirectly into a plant cell to the effect that uptake of the polynucleotide sequence by the said plant cell is achieved, whereby the genotype of said plant cell is modified. Transformation regularly involves the exposure of plant cells in culture, or more or less organised in a a tissue or callus phase, by incubating cells or tissue with so-called 'naked' polynucleotide sequences, bombardment of cells or tissue with microprojectiles carrying the polynucleotide sequence, microinjecting a solution containing the polynucleotide sequence into cells or tissue, incubating or contacting a plant cell or tissue with bacteria or viruses capable of transferring a polynucleotide sequence to the plant and the like.

Transformation methods that may be used include but are not limited to the calcium/polyethylene glycol method for protoplasts (Krens, F.A. et al., 1982, Nature 296, 72-74; Negrutiu I. et al, June 1987, Plant Mol. Biol., 10-19), electroporation of protoplasts (Shillito R.D. et al., 1985 Bio/Technology 3, 1099-1102), microinjection into plant

material (Crossway A. et al., 1986, Mol. Gen. Genet. 202, 179-185), (DNA or RNA-coated) particle bombardment of various plant material (Klein T.M. et al., 1987, Nature 327, 70), infection with viruses and the like.

In a preferred embodiment of the invention use is made of Agrobacterium-mediated DNA transfer. Especially preferred is the use of the so-called binary vector technology as disclosed in EP-A 120 516 and U.S. Patent 4,940,838).

After administering the transforming polynucleotide(s) to the plant material using any of the above methods, transformed cells obtained and selected according to the invention may be used as such, for instance for the production of a pharmaceutical compound in cell suspension cultures. Transformed plant cells may also be used to generate a whole new plant. The available method is itself not critical to the invention as long as uptake of the administered genetic material into the plant cell and integration of (a copy) of the genetic material into the genome of the plant cell is obtained, and the said plant material is amenable to regeneration into a whole new plant. The choice of the technique will depend on the particular type of plant material used, and the preference of the skilled worker.

Especially preferred as plant material are leaf-discs which can be readily transformed and have good regenerative capability (Horsch R.B. et al., (1985) Science 227, 1229-1231).

The use of the marker gene according to the invention is not limited to any particular transformation or regenaration method, although the optimal conditions may have to be determined for each different method used.

5) the polynucleotide sequence of interest

Often the polynucleotide sequence of interest comprises a gene encoding a protein, and the necessary regulatory sequences such that upon expression of the gene the protein is produced in a plant or plant cell at the desired stage and

at the desired site in the plant. The polynucleotide sequence of interest may also comprise genes which can be expressed in the form of an RNA sequence which does not encode protein, such as antisense genes, ribozyme genes and the like. The polynucleotide sequence of interest not necessarily needs to be capable of being transcribed; it may as well be a recognition sequence that can be recognized by proteins, e.g. a recombinase, a nuclease and the like, or by man, serving as a genetic label.

More specific examples of plant expressible genes of interest include, but are not limited to, those that give rise to fungal resistance (International Patent Application WO90/07001; EP-A 440 304), insect resistance (EP-A 159 884), nematode resistance (EP-A 352 052), virus resistance (EP-A 223 452), altered carbohydrate composition (WO90/12876; EP-A 438 904), altered oil composition (EP-A 225 377), seed storage proteins with altered amino acid composition (EP-A 208 418), male sterility (EP-A 329 308), modified flower color (EP-A 335 451), delayed fruit ripening (WO91/01375), salt resistance (WO91/06651), herbicide resistance (EP-A 218 571; EP-A 369 637), production of pharmaceutical products (EP-A 436 003), production of enzymes that can be used in industrial processes and the like.

Usually the process of transformation is eventually followed by identifying the cells having obtained the functional polynucleotide sequence.

The identification of cells having obtained the functional polynucleotide sequence can be done in several ways depending on the inherent property of the functional polynucleotide sequence. If the functional polynucleotide sequence is not expressed in the form of a RNA sequence or a protein an evaluation of the presence of the functional polynucleotide sequence may be done using a hybridisation technique; e.g. Southern blotting, or PCR analysis on genomic DNA. If the functional polynucleotide sequence is expressed in the form of an RNA molecule the presence of such molecule may be determined using inter alea a hybridisation technique

referred to as Northern blotting. If the functional polynucleotide sequence is expressed in the form of a protein a technique referred to as Western blotting can be used. If the protein has an enzymatic activity its presence may be analysed using an enzyme test. In some cases the presence of the polynucleotide sequence of interest need not be analysed on the molecular level, but may be concluded from the phenotype of the transformed plant; e.g. enhanced disease resistance, altered flower color, male-sterility, herbicide resistance, and the like. In the case of herbicide resistance the presence of the polynucleotide sequence of interest can, if desired, be selected for.

The findings that led to the present invention are outlined below for purposes of illustration.

A binary vector was constructed containing a tdc cDNA from Catharanthus roseus under control of the CaMV 35S promoter as an example of a marker polynucleotide sequence and the nptII gene from E. coli under control of the nos promoter as an example of a polynucleotide sequence of interest; pTDCs. This binary vector was used in Agrobacterium tumefaciens mediated transformation of Nicotiana tabacum, via a leaf-disc transformation procedure.

Using 4-methyltryptophan (4-mT) as selective compound it was found that explants of tobacco plants transformed with pTDCs survived selection, gave rise to shoot formation and could be regenerated into whole plants on a medium containing up to 0.5 mM 4-mT. Explants of tobacco plants that did not contain the pTDCs construct (control plants) turned pale and eventually died. Virtually no explants of the control plants escaped from selection.

To check for the presence of the polynucleotide sequence of interest the surviving shoots were transferred to a medium containing 100 mg/l kanamycin. All transferred shoots showed normal growth and rooting on this medium indicating that the plants did not only contain the marker but also the nptII gene. This was confirmed on Northern blots which showed that the mRNAs from both genes were present in the 4-mT resistant

cells.

These results exemplify that a tryptophan analogue can be used as selective compound in the selection of plants or plant cells having obtained a polynucleotide sequence of interest, using a gene encoding a tryptophan analogue converting activity as a marker. It should be understood that although the plant expressible nptII gene can be used as a selectable marker gene for plant transformation it was used as a typical plant expressible gene of interest. Thus, it will be appreciated by those of skill in the art that in principle any plant expressible gene, including those that are not selectable marker genes, can be introduced into a plant cell similar as illustrated for the npt gene in the this example. Examples of such plant expressible genes of interest have been referred to above together with the references where their isolation and characteristics were described.

Although the use of the tryptophan analogue converting activity as a marker has been illustrated in more detail for genetic transformation, those skilled in the art will appreciate that the selectable marker according to the invention can also be used in classical breeding and for the production of hybrid varieties and so forth.

All references cited in this specification are indicative of the level of skill in the arts to which the invention pertains. All publications, whether patents or otherwise, referred to previously or later in this specification are herein separately incorporated by reference.

The Examples given below are just given for purposes of enablement and do not intend in any way to limit the scope of the invention.

EXPERIMENTAL

Tryptophan decarboxylase assay

Tryptophan decarboxylase activity was determined according to Pennings E.J.M. et al., (1987) Anal. Biochem. 165, 133-136. Protein determinations were performed according to

Bradford, M.M. et al., (1976) Anal. Biochem. 72, 248.

Purification of TDC isolated from Catharanthus roseus cell suspension cultures

Cell suspensions of C. roseus were grown for 5 days on induction medium (Knobloch K.H. and Berlin J. (1980) Z. Naturforsch. 35c, 551), harvested, frozen in liquid nitrogen and either stored at -80°C or used immediately for purification of TDC. Partially purified TDC (estimated purity about 20%) was obtained after ammonium sulphate precipitation, anion exchange and size exclusion chromatography as described by Pennings, E.J.M. et al., (1989) Journal of Chromatography 483, 311-318). After size exclusion chromatography, fractions containing TDC activity were pooled. The fractions were adjusted to Laemmli sample buffer conditions and boiled for 5 min. The samples were subjected to preparative SDS PAA gel electrophoresis on 3 mm thick discontinuous gels consisting of a 3.75% stacking gel and a 10% separation gel according to Laemmli, U.K. et al., (1970) Nature 227, 680-685. After electrophoresis, gels were stained for 90 min. in 10% acetic acid, 40% methanol, 0.1% coomassie brilliant blue R250 and destained for 60 min. in 10% acetic acid, 10% methanol. The 47kD TDC monomer band was sliced out of the gel and soaked in water. Electroelution of the TDC protein from the gel fragments was performed according to Hunkapillar M.W. et al, (1983) Methods in enzymology 91, 227-236). Recovery of the TDC protein after electrophoresis and electroelution as determined by analytical SDS PAA gel electrophoresis was estimated to be 90%.

Preparation of antiserum against TDC

Eluate samples containing 150 µl of Freund's complete adjuvant (Gibco) were injected subcutaneously in two New Zealand white rabbits. Booster injections, eluate samples containing 75 µg of denatured TDC emulsified with 600 µl of Freund's incomplete adjuvant, were administered three times

at two-week intervals. The rabbits were bled two weeks after the last injection. Blood samples were left at room temperature for several hours and centrifuged at 2000 rpm for 30 minutes. The clear supernatant was harvested and stored at -20°C. Antibody titers were determined by Enzyme Linked Immunosorbent Assay (ELISA).

ELISA

Wells of a Dynatech microtiter plate were coated overnight at 4°C with 0.1 µg of purified denatured TDC in 100 µl 0.05 M carbonate buffer pH 9.6. After rinsing, plates were incubated for 1 hour with phosphate buffered saline (PBS: 140 mM NaCl, 20 mM Na-phosphate pH 7.4) containing 1% gelatin and 0.05% Tween 20 at 37°C and rinsed again. Antiserum diluted in PBS/gelatin/Tween was added and incubated for 2 hours at 37°C. The wells were rinsed again and goat anti-rabbit globulins conjugated with alkaline phosphatase (Sigma A8025), 1000 fold diluted in PBS/gelatin/Tween was added. Following incubation for 2 hours at 37°C and rinsing, 0.5 mg/ml of the substrate paranitrophenylphosphate dissolved in 10% diethanolamine pH 9.8 was added. The reaction was stopped by adding 1 volume of 1 N NaOH. The absorbance at 405 nm was measured in a Titertek multiscan photometer.

Plant Material

A. Catharanthus roseus

Cell suspension cultures of C. roseus L. (G. Don) were grown in LS medium (Linsmayer et al., (1965) Physiologica plantarum 18, 100-127) containing 2 mg/l 1-naphthalene acetic acid (NAA), 0.2 mg/l kinetin (KIN) and 0.03% w/v sucrose at 27°C on a Kühner Lab-shaker with a shaking diameter of 5 cm at 95 rpm under a 12 hour light/dark regime. Subculturing was performed every 10-12 days by 10-fold dilution of the cells in fresh medium. Induction of TDC activity was achieved by transferring 10 days old cells to induction medium (IM) as described by Berlin et al. (Berlin J. et al., (1983) Z. Naturforsch. 38C, 346).

Hairy root cultures of C. roseus were subcultured every week in modified Gamborg B5 medium (Gamborg O.L. et al., (1968) Exp. Cell. Res. 50, 151-158). The concentration of macronutrients and the $CaCl_2$ concentration were lowered respectively four and two times.

Catharanthus seeds (Vinca rosea, variety Morning mist) were obtained from Kieft (Blokker, Holland), and grown in the greenhouse at 23°C under a 12 hour light/dark regime.

Nicotiana tabacum

Nicotiana tabacum, cv Petit Havanna SR1 plants were grown in vitro on solidified MS medium (Murashige, T. et al., (1962) Physiol. Plant. 15, 473-497) containing 30g/l sucrose at 27°C with 12 hours illumination each day.

DNA-methodology

DNA isolation, subcloning, restriction analyses and sequencing were performed using standard procedures well known to persons skilled in the art, vide e.g. Maniatis et al., 1982. Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Northern blot analysis

Total RNA from green tissues of in vitro plants was isolated according to Van Slogteren, G.M.S. et al., (1983) Plant Molecular Biology 2, 321-333. RNA was glyoxylated, electrophoresed on 1.5% agarose gels and transferred to Genescreen membranes using the capillairy blot method. The cloned tdc cDNA was ^{32}p labeled with random primers (Prime-it kit, Stratagene) and hybridized to the blot in 5x SSPE, 50% formamide, 0.5% SDS at 42°C. After 60-65 hours, filters were washed in 0.1xSSPE, 5% SDS at 65°C for 15 min. and once in 0.5xSSPE at room temperature for 5 min. Hybridization was visualized by exposing the RNA blots at -80°C for 1-3 days to Fuji-RX films mounted on Kyokko-LHII intensifying screens.

DNA, RNA and protein sequencing

Deletions of the EcoRI inserts of pCCR2 and pCCR19 were generated from both 5' and 3' ends using the ExoIII/Mung-bean system (Promega). The resulting deletion constructs were completely sequenced by the dideoxy chain termination method (Sanger, F. et al., (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467). Both strands were sequenced over their entire length. RNA sequencing was performed according to Geliebter, J. et al., (1987) Focus 9:1, 5-8). Sequence data were analysed using the University of Wisconsin Genetics Computing Group programs.

Protein sequencing was performed at the Max Planck Institut für Molekulare Genetic in Berlin.

Tobacco leaf-disc transformation

The leaf-disc transformation procedure was essentially as described by Horsch et al., supra. Nicotiana tabacum SR1 leaf-discs were incubated for 20 minutes in 90 ml MS 10 medium (MS 10: MS medium containing 0.1 mg/l naphthalene acetic acid (NAA) and 1.0 mg/l benzylaminopurine (BAP)) supplemented with 10 ml of Agrobacterium tumefaciens strain LBA 4404 grown overnight in LC (Maniatis et al., 1982, supra), harbouring the binary vectors with the various DNA constructs. Leaf explants were blotted dry and placed upside down on MS 10 medium. After 48 hours, explants were transferred to MS 10 medium containing 100 mg/l cefotaxime, 100 mg/l vancomycin and 100 mg/l kanamycin or 0-1mM 4-methyl tryptophan for selection of transgenic shoots.

EXAMPLE 1

Isolation of a cDNA clone encoding tryptophan decarboxylase

A Construction of a lambda-gt11 expression cDNA library

Total RNA was isolated from a suspension culture of Catharanthus roseus cultured on induction medium for 24 hours. Poly A^+ RNA was isolated as described by van Slogteren et al. (1983), supra. with a minor modification; binding buffer was used to wash loaded oligo (dT)-cellulose columns.

First strand cDNA synthesis was as described by Maniatis et al. (1982), supra. Second strand synthesis was according to Gubler and Hoffman (1983) Gene 25, 263-269) with ommision of DNA ligase and ß-NAD. Phosphorylated EcoRI linkers (Pharmacia) were ligated to double stranded (ds) cDNA according to Maniatis et al., (1982), supra. Linked ds cDNA was EcoRI digested, size selected by chromatography on Sepharose CL-4B and cloned in the EcoRI site of lambda-gt11 (Promega). After packaging (Promega packaging mix) and infection of the host strain E. coli Y1090 (Promega, O.D. ≈0.6, grown in LC containing 0.2% maltose and 10mM $MgSO_4$) $2.32x10^5$ pfu/µg lambda-gt11 were obtained of which 82% were recombinant. The cDNA library was amplified according to Huyng, T.V. et al., (1985) DNA Cloning, a practical approach 1, 49-78.

B. Screening of the cDNA library

After amplification, $2x10^5$ plaques were screened with polyclonal antiserum raised against denatured TDC. Phages absorbed to Escherichia coli Y1090 were plated in 8 ml top-agarose on LC medium in 14,5 cm diameter petridishes and incubated at 37°C. A maximum of 20.000 plaques were plated on each petridish. After 3.5 hours (no plaques were visible), nitrocellulose filters (Schleicher and Schuell) saturated with 10mM IPTG (air-dryed) were placed on top of the agarose. The plates were incubated for another 3.5 hours and placed overnight at 4°C. After marking the position with a needle and ink, filters were lifted from the agarose (now plaques were visible) and washed extensively in TBST (10mM Tris-HCl pH8.0, 150mM NaCl, 0.05% Tween 20) to remove agarose remnants.

To saturate nonspecific protein binding sites, filters were incubated for 30' in TBST containing 1% gelatin. The filters were transferred to a solution containing 1000 fold diluted TDC antiserum in TBST and incubated overnight at room temperature. The antiserum was preincubated with 0.5 mg/ml Y1090 protein extract to reduce the background produced by

anti E. coli antibodies. After rinsing the filters in TBST (three times for 15') indubation continued for 60' in 1:7500 diluted second antibody alkaline-phosphatase conjugate. Again the filters were rinsed in TBST followed by a color reaction, performed according to Promega (Protoblot Immunoscreening System, technical manual). This reaction was stopped by replacing the substrate solution with 10mM Tris, 1 mM EDTA pH 8.0.

C. Characterization and sequence determination of the isolated cDNA clones

Screening of about 200,000 initial transformants resulted in the isolation of 7 positive clones. Purification of the lambda phages and isolation of their insert revealed, that 5 clones contained an insert of about 1600 bp and 2 clones contained an insert of about 830 bp; both types of inserts did not cross hybridize. The inserts were subcloned in the EcoRI site of a bluescript SK vector (Stratagene) resulting in pCCR2 (ca. 1600 bp insert) and pCCR19 (ca. 830 bp insert). Northern blot analysis revealed, that the inserts of pCCR2 and pCCR19 correspond both with a mRNA of approximately 1700 nucleotides, a size expected for tdc mRNA.

Upon Northern blot analysis, it was observed that levels of mRNA hybridising to pCCR2 were raised in cell suspensions from Catharanthus roseus grown on induction medium as compared to cells grown in non-induction medium; no differences were observed using the pCCR19 insert as probe. This induction is in accordance with the de novo synthesis of tryptophan decarboxylase in cell suspensions of C. roseus as described by Noé, W. et al., (1985) Planta 166, 500-504). Hence, we concluded that the insert of pCCR2 could correspond with a tdc cDNA.

Both strands of the pCCR2 insert were sequenced using subclones and deletions generated by ExoIII and Mung bean nuclease digestions (Promega).

The pCCR2 insert contained an open reading frame lacking a startcodon, indicating that an incomplete cDNA clone had been

obtained. The missing sequences were determined by primer extension on poly A$^+$ RNA (See Experimental Part of this specification). The determined nucleotide sequence is given in SEQIDNO: 1. This sequence reveals two putative translation start codons; the ATG starting at position 60 (first ATG) and the ATG starting on position 90 (second ATG) as indicated in SEQIDNO: 1.

N-terminal sequence analysis of the purified 47kD TDC monomer revealed that the isolated protein starts with the amino acid sequence: Ser-Pro-Val-Gly-Glu-Phe-Lys-Pro-Leu, corresponding with nucleotide position 99 to 125 in SEQIDNO: 1. Since both ATG codons are in frame and we did not know whether the mRNA is translated from the first or the second ATG we decided to use the second ATG as translation start codon in our constructs.

To complete our tdc cDNA fragment we set out to synthesise an oligonucleotide encoding the missing amino acid residues spanning nucleotide position 90 to 125 (SEQIDNO: 1), flanked by a SalI and EcoRI restriction site, and an artificially introduced NcoI site. We finally obtained the oligonucleotide sequence as depicted in SEQIDNO: 2.

This oligonucleotide sequence contains a point mutation corresponding with position 93 in SEQIDNO: 1, creating a NcoI site; the point mutation changes codon 2 from TCC encoding a Serine residue into GCC encoding a Alanine residue. Due to an error in DNA synthesis also codon 10 (AAG) encoding a Lysine residue was changed into AAT encoding an Asparagine residue.

EXAMPLE 2

Construction of the binary vectors pBDH5, pTDCs and pTDCa

The wide host range expression vector pBDH5 was constructed by deleting the SalI restriction site from a Bin19 binary vector (Bevan, M. et al., (1984) Nuc. Acid Res. 12, 8711-8721) and inserting a 35S CaMV expression cassette from pDH51 (Pietrzak, M. et al., (1986) Nuc. Acid Res. 14, 5857-5868) as an EcoRI fragment (figure 1). The obtained construct in which the CaMV 35S promoter has the same

orientation as the nos promoter of the nptII gene (pBDH5) was used in further cloning procedures.

The synthetic SalI - EcoRI fragment depicted in SEQ ID NO: 2 was cloned in pIC20H (Marsch, J.L. et al., (1984) Gene 32, 481-485). The incomplete tdc cDNA fragment of pCCR2 was cloned as EcoRI fragment, using the EcoRI in the coding region starting at position 135 in SEQIDNO: 1, behind the synthetic SalI-EcoRI fragment, yielding pIST6 (Figure 1). The completed tdc cDNA was excised as SalI - XhoI fragment (vide SEQ ID NO: 3) and cloned in both orientations in the SalI site of the pBDH5 binary vector resulting in pTDCs (sense construct) and pTDCa (antisense construct) (figure 1). The binary vectors were electroporated to Agrobacterium tumefaciens strain LBA 4404 as described by Mattonovich, D. et al., (1989) Nuc. Acid Res. 17, 6747) resulting in LBA4404 (pBDH5), LBA4404 (pTDCs) and LBA4404 (pTDCa) respectively.

EXAMPLE 3

Generation of tdc transgenic plants; Kanamycin selection

Leaf-disc transformation of Nicotiana tabacum and subsequent selection on 100 mg/l kanamycin resulted in transgenic plants harbouring T-DNA (transferred DNA) derived from pBDH5 (empty vector), pTDCs (sense construct) and pTDCa (antisense construct) tdc gene constructs, hereinafter referred to as pBDH5, pTDCs and pTDCa respectively. Levels of tdc mRNA were determined in green tissues of the obtained transgenic plants. Plants harbouring pTDCs displayed at least a 10-fold variation in tdc mRNA accumulation between the best (tobacco line E) and the worst expressors (lines A and G) (figure 2). Longer exposure times of the blot clearly show the presence of tdc mRNA in the plants A and G. Four out of ten plants (B, C, D and F) had an intermediate level of tdc mRNA, while the same number of plants showed a strong expression (E, H, I and J). No tdc mRNA could be detected in plants harbouring the pBDH5 and pTDCa plasmids.

Of 5 plants (A, B, E, F and J) harbouring the pTDCs constructs TDC activities were determined (Table 1). These

data show that the overexpression of tdc cDNA in tobacco tissues results in TDC activity, which is normally not present in tobacco plants. Plant E showing the highest tdc mRNA level also showed the highest TDC enzyme activity.

Table 1: TDC activities in tdc transgenic Nicotiana tabacum plants.

		TDC activity pkat/mg protein
SENSE	A	4
	B	5
	E	69
	F	5
	J	19
ANTISENSE		0
VECTOR		0

Table 1:TDC activities, as determined by HPLC analysis, in pBDH5 (vector), pTDCa (antisense) or pTDCs (sense) transformed N. tabacum plants.

EXAMPLE 4

Sensitivity of leaf explants from tdc transformed tobacco plants to 4-methyl tryptophan

To determine the sensitivity of the tdc transgenic Nicotiana tabacum plants for 4-methyl tryptophan, leaf explants were incubated on shooting medium (MS 10) containing 0, 0.05, 0.1, 0.5 and 1 mM 4-methyl tryptophan. Explants of three tdc transgenic tobacco plants were tested (pTDCs; A, C

and I) displaying low, intermediate and high tdc transcript levels respectively. As a control, leaf explants from pBDH5 transformed plants were tested. After 6 weeks, shooting of the explants was scored relative to the amount of shooting obtained on medium without 4-methyl tryptophan.
Explants from the pTDCs-I (high tdc transcript level) transformed plants gave rise to shoot formation on medium containing up to 0.5 mM 4-mT although some reduction in shooting frequency was observed at this concentration (figure 2). Explants of pTDCs-A (low tdc transcript level) gave rise to shoot formation up to 0.1 mM 4-mT. Higher concentrations of 4-mT resulted in reduced or absence of shooting.
The plant with an intermediate tdc mRNA expression (pTDCs-C) also showed intermediate sensitivity to 4-mT. From our experiments we observed that there seems to be a better correlation between tdc mRNA levels and sensitivity to 4-mT as opposed to TDC enzyme levels and sensitivity to 4-mT. We therefore concluded that the enzyme assay is not very sensitive; only large differences in TDC enzyme levels allow to predict differences in sensitivity to 4-mT.

When cultured on 1mM 4-mT, none of the explants harbouring the tdc sense construct gave rise to substantial shoot formation.

Nearly all explants of the pBDH5 transformed plants turned pale and died on medium containing 4-mT. Some explants cultured on 0.05 and 0.1 mM 4-mT gave rise to a few "escape" shoots. Those shoots predominantly arose from explants containing main-nerve tissue.

EXAMPLE 5

Generation of transgenic plants using tdc as marker and selecting on 4-methyl tryptophan

Based on the shooting capacity of tdc transgenic leaf-discs on 4-mT containing MS 10 medium, an experiment was performed using tdc as selection marker in Nicotiana tabacum leaf-disc transformation. Leaf explants of N. tabacum SR1 were cocultivated with LBA 4404 containing the tdc/nptII

constructs. After 48 hours leaf-discs were incubated on MS 10 medium containing 0.1 mM 4-mT. After 6 weeks the explants were scored for shoot formation.

Leaf explants incubated with LBA 4404 (pTDCs) gave rise to abundant shoot formation on medium containing up to 0.1 mM 4-mT (figure 4). Explants cultured on medium containing 0.5 mM 4-mT showed a reduced and delayed shooting response. No shoot formation was observed on medium containing 1 mM 4-mT.

Control plates, containing explants treated with LBA4404 (pTDCa) (antisense), LBA4404 (pBDH5) or leaf-discs not treated with Agrobacterium at all, did not give rise to substantial shoot formation. Some "escape" shoots were formed out of main nerve tissue from explants cultured on 0.05 and 0.1 mM 4-mT as expected from our data.

All the obtained shoots from leaf explants cultured on MS 10 medium supplemented with 0.1 mM 4-mT were excised and grown on MS medium containing 0.1 mM 4-mT. On further growth, all plants derived from leaf-disc transformation without Agrobacterium or with LBA 4404 containing pBDH5 and pTDCa turned pale and died. Of 30 transgenic shoots derived from the transformation of leaf-discs with LBA 4404 containing pTDCs, 24 shoots (80%) showed normal growth on medium containing 0.1 mM 4-mT. Six shoots died on this medium, which is in accordance with the number of escape shoots formed on the control plates. In our hands, these data were comparable to those obtained with leaf-disc transformation experiments using kanamycin as selective agent.

To establish whether the nptII gene was transferred to the plant cells, the surviving shoots were transferred to medium containing 100 mg/l kanamycin. All transferred shoots showed normal growth and rooting on this medium suggesting the plants to be transformed with both the tdc gene and the nptII gene. Transcript levels of both genes were determined in 6 shoots by Northern blot analysis (figure 4). It appeared that the nptII gene was transferred and expressed to the plant.

From these data we conclude that 4-mT can be used as a selective agent for the efficient selection of transformed

plant cells using the tdc gene as a marker gene.

SEQUENCE LISTING

INFORMATION FOR SEQ ID NO: 1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1731 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA to mRNA
(iii) ORIGINAL SOURCE:
(A) ORGANISM: Catharanthus roseus
(B) STRAIN: G. don
(D) DEVELOPMENTAL STAGE: Suspension cells
(iv) IMMEDIATE SOURCE:
(A) LIBRARY: lamba gt11
(B) CLONE: pCCR2
(v) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 60..1559
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(A) NAME/KEY: polyA_signal
(B) LOCATION: 1708..1713
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                                                                       155
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(A) LENGTH: 61 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA to mRNA
(iii) HYPOTHETICAL: YES
(iv) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: synthetic oligonucleotide
(v) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 11..61
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(B) LOCATION: 56..61
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(viii) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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(C) STRANDEDNESS: double
(D) TOPOLOGY: both
(ii) MOLECULE TYPE: cDNA to mRNA
(iii) HYPOTHETICAL: YES
(iv) ORIGINAL SOURCE:
(A) ORGANISM: Catharanthus roseus
(B) STRAIN: G. don
(D) DEVELOPMENTAL STAGE: Suspension cells
(v) IMMEDIATE SOURCE:
(A) LIBRARY: lamba gt11
(B) CLONE: pCCR2
(vi) FEATURE:
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(viii) FEATURE:
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(ix) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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             65                  70                  75

                                                                       289
GGA ATG ACA AAT TGG ATG AGC CCT AAT TTT TAT GCA TTT TTT CCT GCC
Gly Met Thr Asn Trp Met Ser Pro Asn Phe Tyr Ala Phe Phe Pro Ala
         80                  85                  90

                                                                       337
ACT GTT AGT TCA GCT GCC TTT TTA GGA GAA ATG TTG TCT ACT GCC CTA
Thr Val Ser Ser Ala Ala Phe Leu Gly Glu Met Leu Ser Thr Ala Leu
     95                  100                 105

                                                                       385
AAT TCA GTA GGC TTT ACT TGG GTT TCT TCA CCA GCC GCC ACC GAA TTA
Asn Ser Val Gly Phe Thr Trp Val Ser Ser Pro Ala Ala Thr Glu Leu
110                 115                 120                 125

                                                                       433
GAA ATG ATT GTT ATG GAT TGG TTG GCT CAG ATC CTT AAA CTC CCC AAA
Glu Met Ile Val Met Asp Trp Leu Ala Gln Ile Leu Lys Leu Pro Lys
                130                 135                 140

                                                                       481
TCT TTC ATG TTT TCA GGT ACC GGT GGC GGC GTC ATC CAA AAC ACC ACT
Ser Phe Met Phe Ser Gly Thr Gly Gly Gly Val Ile Gln Asn Thr Thr
            145                 150                 155

                                                                       529
AGC GAG TCC ATT CTT TGT ACA ATC ATT GCC GCC CGG GAA AGG GCC CTG
Ser Glu Ser Ile Leu Cys Thr Ile Ile Ala Ala Arg Glu Arg Ala Leu
        160                 165                 170

                                                                       577
GAG AAG CTC GGT CCC GAT AGT ATT GGA AAA CTT GTC TGT TAC GGA TCC
Glu Lys Leu Gly Pro Asp Ser Ile Gly Lys Leu Val Cys Tyr Gly Ser
    175                 180                 185

                                                                       625
GAT CAA ACC CAT ACC ATG TTC CCC AAA ACT TGC AAA TTG GCG GGA ATT
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Asp Gln Thr His Thr Met Phe Pro Lys Thr Cys Lys Leu Ala Gly Ile
190                 195                 200                 205

TAT CCG AAT AAT ATT AGG TTA ATA CCT ACG ACC GTC GAA ACG GAT TTC      673
Tyr Pro Asn Asn Ile Arg Leu Ile Pro Thr Thr Val Glu Thr Asp Phe
                210                 215                 220

GGC ATC TCA CCT CAA GTT CTA CGA AAA ATG GTC GAG GAT GAC GTG GCG      721
Gly Ile Ser Pro Gln Val Leu Arg Lys Met Val Glu Asp Asp Val Ala
            225                 230                 235

GCC GGA TAT GTA CCG CTG TTC TTA TGC GCT ACC CTG GGT ACC ACC TCG      769
Ala Gly Tyr Val Pro Leu Phe Leu Cys Ala Thr Leu Gly Thr Thr Ser
        240                 245                 250

ACC ACG GCT ACC GAT CCT GTG GAC TCA CTT TCT GAA ATC GCT AAC GAG      817
Thr Thr Ala Thr Asp Pro Val Asp Ser Leu Ser Glu Ile Ala Asn Glu
    255                 260                 265

TTT GGT ATT TGG ATC CAC GTG GAT GCT GCT TAT GCG GGA AGC GCC TGT      865
Phe Gly Ile Trp Ile His Val Asp Ala Ala Tyr Ala Gly Ser Ala Cys
270                 275                 280                 285

ATA TGT CCC GAG TTT AGA CAT TAC TTG GAT GGA ATC GAA CGA GTT GAC      913
Ile Cys Pro Glu Phe Arg His Tyr Leu Asp Gly Ile Glu Arg Val Asp
                290                 295                 300

TCA CTG AGT CTG AGT CCA CAC AAA TGG CTA CTC GCT TAC TTA GAT TGC      961
Ser Leu Ser Leu Ser Pro His Lys Trp Leu Leu Ala Tyr Leu Asp Cys
            305                 310                 315

ACT TGC TTG TGG GTC AAG CAA CCA CAT TTG TTA CTA AGG GCA CTC ACT     1009
Thr Cys Leu Trp Val Lys Gln Pro His Leu Leu Leu Arg Ala Leu Thr
        320                 325                 330

ACG AAT CCT GAG TAT TTA AAA AAT AAA CAG AGT GAT TTA GAC AAA GTT     1057
Thr Asn Pro Glu Tyr Leu Lys Asn Lys Gln Ser Asp Leu Asp Lys Val
    335                 340                 345

GTG GAC TTC AAA AAT TGG CAA ATC GCA ACG GGA CGA AAA TTT CGG TCG     1105
Val Asp Phe Lys Asn Trp Gln Ile Ala Thr Gly Arg Lys Phe Arg Ser
350                 355                 360                 365

CTG AAA CTT TGG CTC ATT TTA CGT AGC TAT GGA GTT GTT AAT TTA CAG     1153
Leu Lys Leu Trp Leu Ile Leu Arg Ser Tyr Gly Val Val Asn Leu Gln
                370                 375                 380

AGT CAT ATT CGT TCT GAC GTC GCA ATG GGC AAA ATG TTC GAA GAA TGG     1201
Ser His Ile Arg Ser Asp Val Ala Met Gly Lys Met Phe Glu Glu Trp
            385                 390                 395

GTT AGA TCA GAC TCC AGA TTC GAA ATT GTG GTA CCG AGA AAC TTT TCT     1249
Val Arg Ser Asp Ser Arg Phe Glu Ile Val Val Pro Arg Asn Phe Ser
        400                 405                 410
```

CTT GTT TGT TTT AGA TTA AAA CCT GAC GTT TCG AGT TTA CAT GTA GAA 1297
Leu Val Cys Phe Arg Leu Lys Pro Asp Val Ser Ser Leu His Val Glu
415 420 425

GAA GTG AAT AAG AAA CTT TTG GAC ATG CTT AAC TCG ACG GGA CGA GTT 1345
Glu Val Asn Lys Lys Leu Leu Asp Met Leu Asn Ser Thr Gly Arg Val
430 435 440 445

TAT ATG ACT CAT ACT ATT GTG GGA GGC ATA TAC ATG CTA AGA CTG GCT 1393
Tyr Met Thr His Thr Ile Val Gly Gly Ile Tyr Met Leu Arg Leu Ala
450 455 460

GTT GGC TCA TCG CTA ACT GAA GAA CAT CAT GTA CGC CGT GTT TGG GAT 1441
Val Gly Ser Ser Leu Thr Glu Glu His His Val Arg Arg Val Trp Asp
465 470 475

TTG ATT CAA AAA TTA ACC GAT GAT TTG CTC AAA GAA GCT TGATGAATAA 1490
Leu Ile Gln Lys Leu Thr Asp Asp Leu Leu Lys Glu Ala
480 485 490

GTAAGGGTTT TTTTTTAATT TTTTTTTAAA TTTTATATTT GCTGATTGTT TGAAGAGTTT 1550

AAAAATAAAG TGATTTGTAA AGGTTTATTG TACTCAAACA ATCATGCAAT TAATTATATG 1610

TATTAATTAT GACATGAGAA TAAAATAGAA TTTGTGTGTG CA 1652

CLAIMS

1. A method for the selection of transformed plant cells, comprising the steps of:
i) transforming plant cells with a recombinant polynucleotide comprising a plant expressible gene encoding a tryptophan analogue converting activity,
ii) culturing said plant cells, or growing plant material comprising said plant cells, in the presence of a selective amount of a tryptophan analogue deleterious to non-transformed plant cells, under conditions that allow for the manifestation of said deleterious effect.

2. The method of claim 1, wherein said plant expressible gene encoding a tryptophan analogue converting activity is a plant expressible tryptophan decarboxylase gene.

3. The method of claim 2, wherein the said tryptophan decarboxylase gene is the tdc gene from Catharanthus roseus or a functional derivative thereof.

4. The method of claim 3, wherein said decarboxylase gene is under the control of the CamV 35S promoter.

5. The method of anyone of the claims 1 to 4, wherein said recombinant polynucleotide further comprises a polynucleotide sequence of interest.

6. The method of claim 5, wherein said polynucleotide sequence of interest is a plant expressible gene which gives rise to fungal resistance, insect resistance, nematode resistance, virus resistance, altered carbohydrate composition, altered oil composition, altered amino acid composition, male-sterility, modified flower color, modified fruit ripening, salt resistance, herbicide resistance, antibiotic resistance, production of a secondary metabolite, production of a pharmaceutical protein, or production of an

enzyme that can be used in an industrial process.

7. The method of any one of the claims 1 to 6, wherein the said tryptophan analogue is 4-methyltryptophan.

8. A plant cell obtained by a method of any one of the claims 1 to 7.

9. Plant material harbouring a cell of claim 8.

10. Plant material obtained by growing a plant cell of claim 8.

11. A plant part harbouring a cell according to claim 8, which part is selected from the group consisting of bulbs, flowers, fruits, hairy roots, leaves, microtubers, pollen, roots, seeds, stalks and tubers.

12. A plant regenerated from a cell of claim 8.

13. A plant part derived from a plant according to claim 12, which part is selected from the group consisting of bulbs, flowers, fruits, hairy roots, leaves, microtubers, pollen, roots, seeds, stalks and tubers.

14. A product obtained after the processing of a plant part of claim 13.

15. Use of a tryptophan analogue for the selection of a transformed plant cell.

16. Use of a plant expressible gene encoding an enzyme having a tryptophan analogue converting activity as a marker gene for the selection of transformed plant cells.

17. A method for obtaining a transformed plant comprising the steps of:

i) transforming plant cells with a polynucleotide sequence comprising a plant expressible gene encoding a tryptophan analogue converting activity,
ii) culturing said plant cells, or growing plant material comprising said plant cells, in the presence of a selective amount of a tryptophan analogue deleterious to non-transformed plant cells, under conditions that allow for the manifestation of said deleterious effect,
iii) regenerating surviving cells of step ii) into a plant,
iv) identifying a transformed plant.

18. The method of claim 17, wherein said plant expressible gene encoding a tryptophan analogue converting activity is a plant expressible tryptophan decarboxylase gene.

19. The method of claim 18, wherein the said tryptophan decarboxylase gene is the tdc gene from Catharanthus roseus or a functional derivative thereof.

20. The method of anyone of the claims 17 to 19, wherein said recombinant polynucleotide further comprises a polynucleotide sequence of interest.

21. The method of claim 20, wherein said polynucleotide sequence of interest is a plant expressible gene which gives rise to fungal resistance, insect resistance, nematode resistance, virus resistance, altered carbohydrate composition, altered oil composition, altered amino acid composition, male-sterility, modified flower color, modified fruit ripening, salt resistance, herbicide resistance, antibiotic resistance, production of a secondary metabolite, production of a pharmaceutical protein, or production of an enzyme that can be used in an industrial process.

22. The plant obtained with a method of any one of the claims 17 to 21.

23. Progeny plants obtained after sexually or asexually propagating a plant of claim 12 or 22.

24. A recombinant polynucleotide which can be used for the transformation of plant cells and subsequent selection of transformed plant cells, comprising a plant expressible gene encoding a tryptophan analogue converting activity.

25. The recombinant polynucleotide of claim 24, which further comprises a polynucleotide sequence of interest, with the proviso that said polynucleotide sequence of interest is not a plant expressible nptII gene.

26. The recombinant polynucleotide of claim 24, with the proviso that said polynucleotide sequence of interest is not known as a selectable marker gene for use in the transformation of plants.

27. The recombinant polynucleotide of claim 26, wherein the polynucleotide sequence of interest comprises a plant expressible gene which gives rise to fungal resistance, insect resistance, nematode resistance, virus resistance, altered carbohydrate composition, altered oil composition, altered amino acid composition, male-sterility, modified flower color, modified fruit ripening, salt resistance, production of a secondary metabolite, production of a pharmaceutical protein or production of an industrial enzyme.

28. A recombinant plant DNA genome containing a copy of the recombinant polynucleotide of any one of the claims 24 to 27.

29. A plant or plant cell containing the recombinant plant DNA genome of claim 28.

30. A substantially pure DNA molecule which comprises

the nucleotide sequence represented in SEQIDNO: 2.

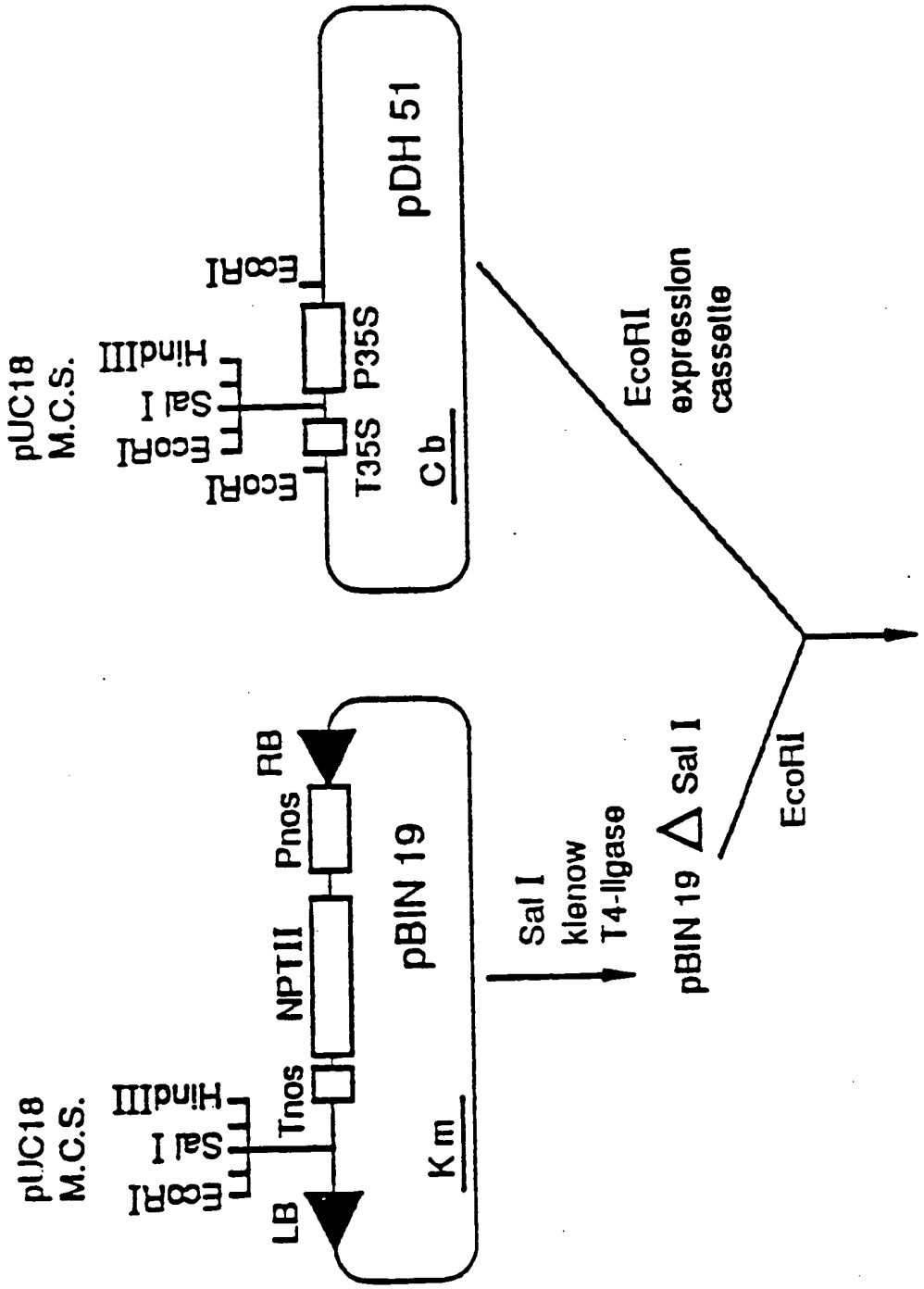
pDH 51
EcoRI
Hind III
Sal I
EcoRI
EcoRI
P35S
T35S
Cb
pUC18
M.C.S.
EcoRI expression cassette
pBIN 19 Δ Sal I
EcoRI
Sal I
klenow
T4-ligase
pBIN 19
RB
Pnos
NPTII
Tnos
LB
Km
Hind III
Sal I
EcoRI
pUC18
M.C.S.

FIGURE 1

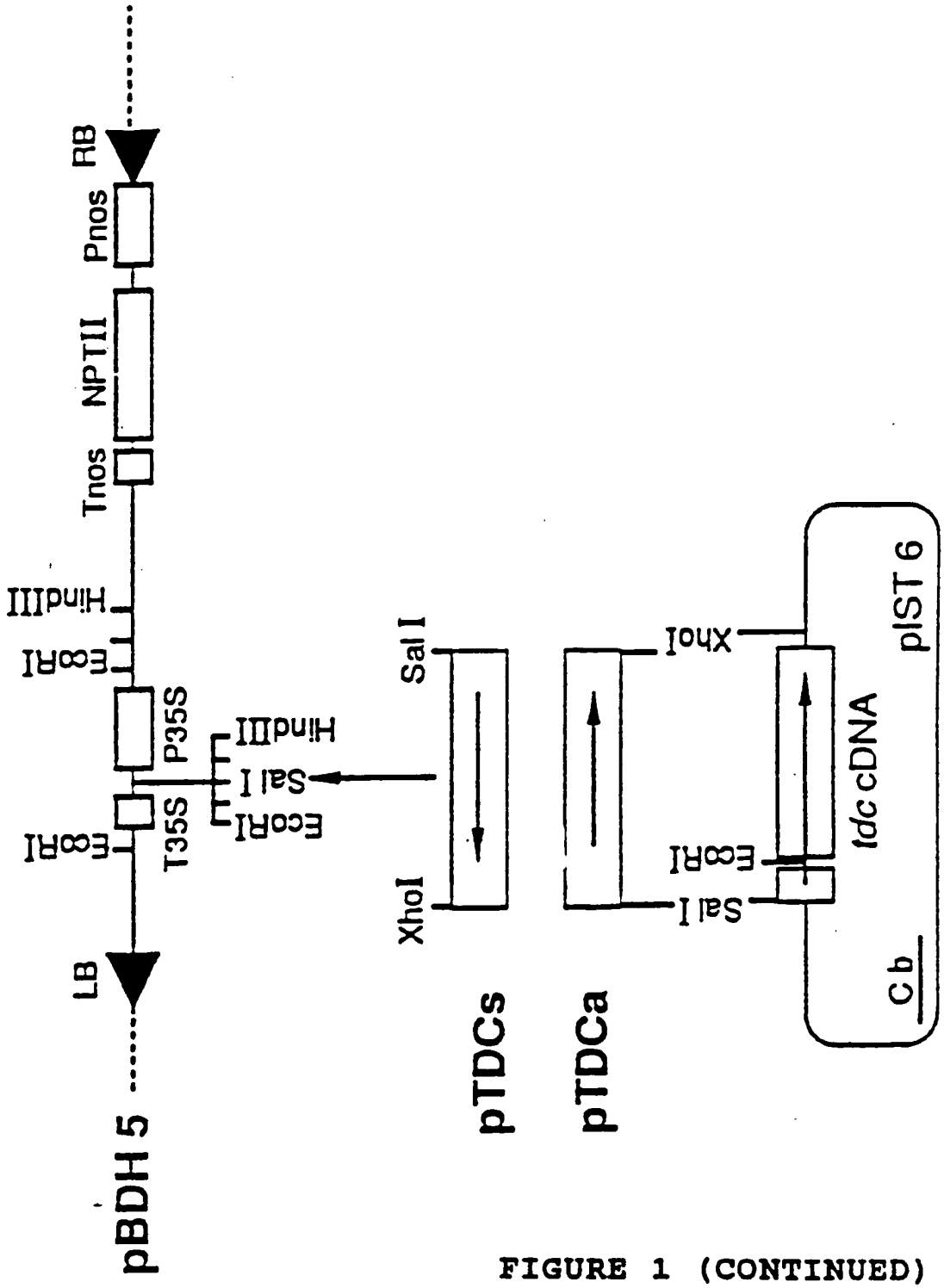

FIGURE 1 (CONTINUED)

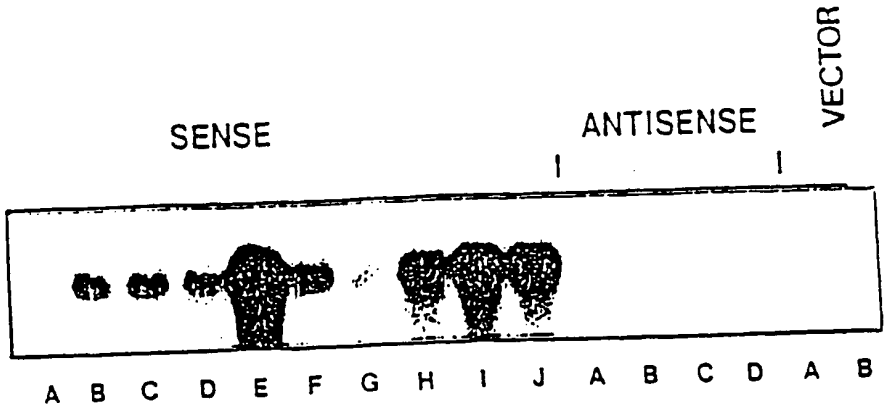

FIGURE 2

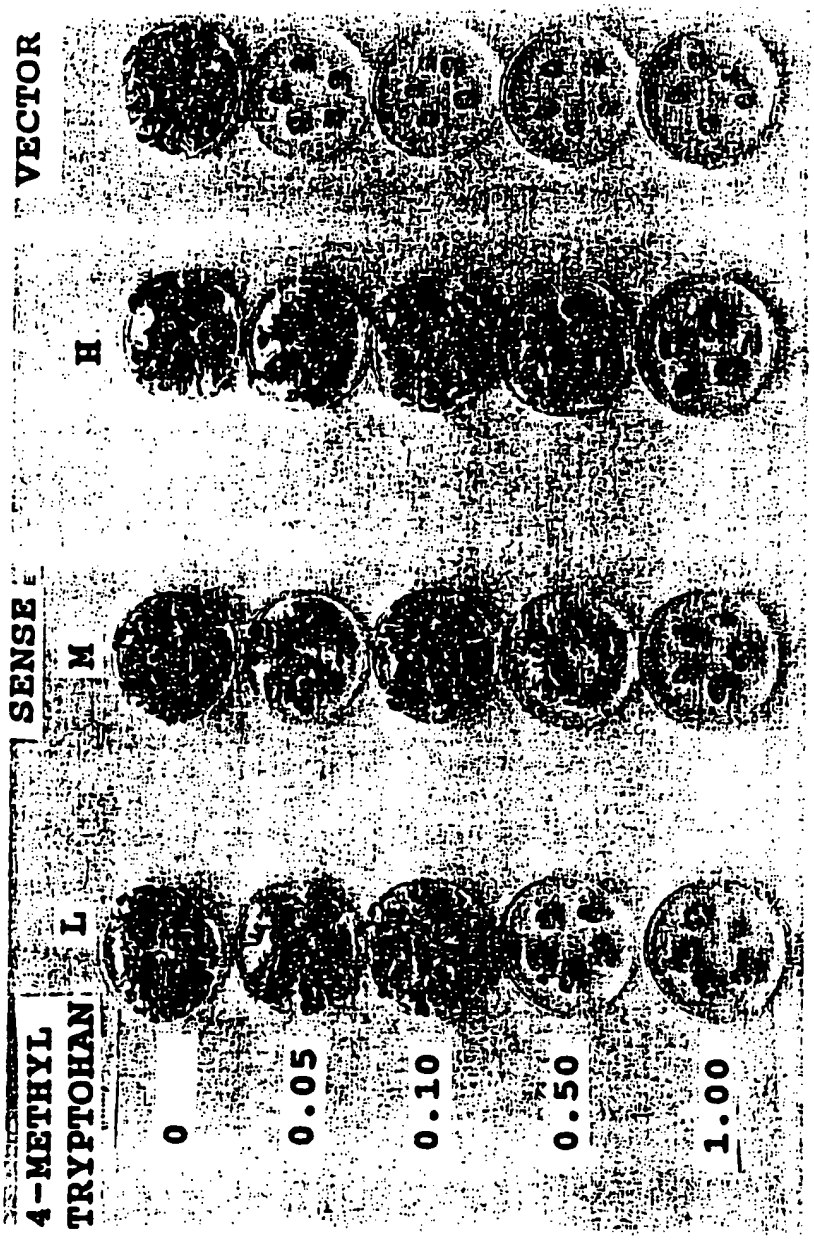

FIGURE 3

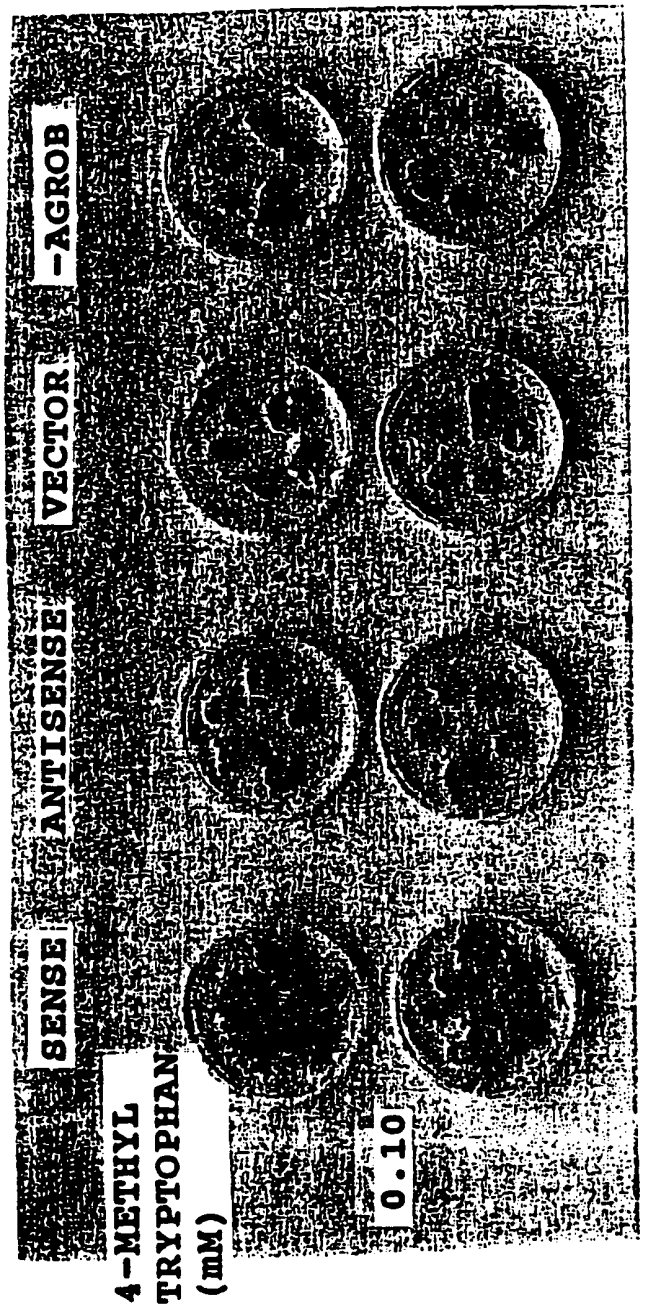

FIGURE 4

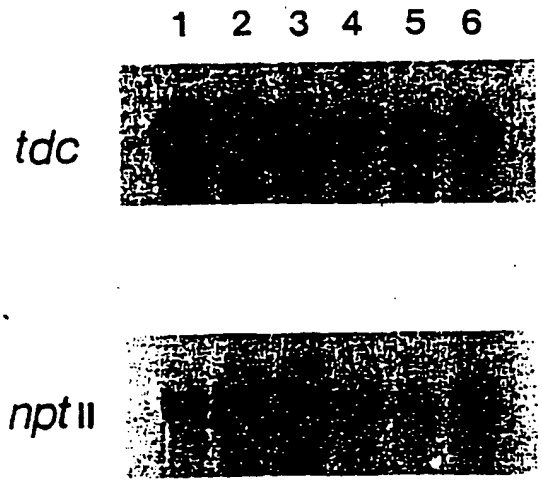

FIGURE 5

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 92/02175

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/82; C12N15/60; C12N5/10; A01H1/02
A01H5/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A01H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	PLANT PHYSIOLOGY. vol. 94, no. 3, November 1990, ROCKVILLE, MD, USA. pages 1410 - 1413 SONGSTAD, D.D., ET AL. 'High levels of tryptamine accumulation in transgenic tobacco expressing tryptophan decarboxylase' cited in the application	7-14, 22-24, 28-29
Y	see the whole document	1-5,7, 16-20
	--- -/--	

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"P" document published prior to the international filing date but later than the priority date claimed

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11 DECEMBER 1992	18. 12. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MADDOX A.D.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	BIOLOGICAL ABSTRACTS vol. 78 , 1984, Philadelphia, PA, US; abstract no. 6552, SASSE, F., ET AL. 'Selection of cell lines of Catharanthus roseus with increased tryptophan decarboxylase activity' cited in the application see abstract & Z. NATURFORSCH. SECT. C BIOSCI. vol. 38, 1983, NO.11/12 pages 916 - 922	1-5,7, 16-20
O,X	J. CELL. BIOCHEM. SUPPL., MEETING HELD JAN. 10-17, 1991. vol. 15A, 1991, page 70 GODDIJN, O.D.M., ET AL. 'Cloning and regulation of the tryptophan decarboxylase gene from Catharanthus roseus' see abstract A320	7-14, 22-24, 28,29
X	EP,A,0 174 791 (MOLECULAR GENETICS) 19 March 1986 see page 20 - page 25	15
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA vol. 86, April 1989, WASHINGTON US pages 2582 - 2586 DE LUCA, V., ET AL. 'Molecular cloning and analysis of cDNA encoding a plant tryptophan decarboxylase: Comparison with animal dopa decarboxylases' see figure 2	30

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 9202175 SA 64335

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 11/12/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0174791	19-03-86	US-A- 4581847	15-04-86
		US-A- 4642411	10-02-87

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